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ACTUAL SURVEY ON TLm (MEDIAN TOLERANCE LIMIT) VALUES OF ENVIRONMENTAL POLLUTANTS, ESPECIALLY ON AMINES, NITRILES, AROMATIC NITROGEN COMPOUNDS AND ARTIFICIAL DYES

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Abstract……Forty-four kinds of organic nitrogen compounds as well as 37 kinds of dyes which contained nitrogen were subjected to TLm test by use of Himedaka (*Oryzias latipes*), and the results were compared with their partition coefficients between *n*-octanol and water.

Concerning organic nitrogen compounds, such good correlation was observed among them that the larger the partition coefficient was, the smaller was the TLm value. Their acute toxicity to fish was supposed to be revealed after their passage through cell membrane of fish to be accumulated in the body. As far as coal-tar dyes and dyestuffs were concerned, there was a correlation between partition coefficient and TLm value. Methylene blue and rose bengale showed strong acute toxicity to fish and it was assumed that high affinity of these dyes to gill was responsible to depressing the function of gill, to make fish suffer from anoxemia and die.

Key words:organic nitrogen compound, coal-tar dye, TLm test, Himedaka (*Oryzias latipes*), *n*-octanol-water partition coefficient.

INTRODUCTION

In Japan, taking the opportunity of PCB poisoning that had occurred in 1968 (usually called "Kanemi Yusho"), it became necessary to evaluate safety of environmental pollutants and almost all the other chemical substances. Examination of chemical substances has been carried out from various points of view since 1973.

Though it is supposed that organic nitrogen compounds among various environ-

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mental pollutants have great influence on human life, few studies have been carried out up to now.

The acute toxicity of 44 kinds of organic nitrogen compounds as well as 37 kinds of nitrogen-containing dyestuffs were investigated in this report. In order to study the effect of environmental pollutants on aquatic living things, there are such methods as carried out by use of protozoa (Kobayashi, 1971), shellfish (Metcalf et al., 1971), fish (Nakamura et al., 1979), and so on. In this report, their acute toxicity to fish were determined by use of Himedaka (*Oryzias latipes*).

Hansch et al. (1964) pursued the correlation between toxicity to fish and chemical structure, and concluded that toxicity of a chemical compound is revealed according to the following two factors: (i) permeability of active substance through membrane which is indicated by the partition coefficient, (ii) binding ability of active substances to some parts of fish which is due to electronic characteristics of the substance. Accordingly, their partition coefficients between *n*-octanol and water were determined to approximate the cellular phases, and it was tried to find out correlation between TLm value and partition coefficient. Moreover, so far as such substances were concerned that had no proportional correlation between TLm values and partition coefficients, it was further attempted to find out the reason of acute toxicity from the standpoint of binding on some parts of fish.

EXPERIMENTS

1. Materials

1・1 Dyestuffs: Light Green SF, Erythrosine, Phloxine and Rose Bengale used were the reference standards of the National Institute of Hygienic Sciences. The other dyes used were commercial products of guaranteed grade.

1・2 *n*-Octanol: Commercial *n*-octanol (95% purity) was washed successively with dilute sulphuric acid, dilute sodium hydroxide solution and distilled water until neutral, then was dried with anhydrous calcium chloride, and distilled through a short fractionation column.

All the other reagents used were of analytical grade.

2. Apparatuses

2・1 Spectrophotometer: Hitachi, type 556, self-registering.

2・2 Gas chromatograph: Yanaco G-80 with flame ionization detection system (Yanagimoto Mfg. Co. Ltd.) equipped with a glass tube of 0.3 cm i. d. ×150 cm in length packed with 20% PEG 20 M+3% NaOH on Celite 545 (40-60 mesh).

2・3 Dissolved oxygen meter: Denki Kagaku Keiki Co. Ltd., type DO-3.

2・4 Coulter counter: Nikkaki, Model ZBI.

3. Method

3・1 Determination of TLm value

3・1・1 Test fish: Himedaka of the same age (about 2 cm in length, 0.2 g in weight) and carp (about 10 cm in length, 30.0 g in weight) were chosen and acclimated for 10 days in the tap water before experiment.

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3・1・2 Test solution : Chemical substances were dissolved in water and neutralized with 0.01 N NaOH or HCl, if necessary. Some substances which were difficult to dissolve in water were dissolved in 1 ml of ethanol, and then diluted with water.

3・1・3 Operating conditions : TLm test was carried out according to the procedure of Japan Industrial Standards (Japanese Industrial Standards Committee, 1971). Ten fish of Himedaka per one trial were kept in 2 liter of deionized water at 25°C and, after 24 or 48 hours, lethal concentration of 50% fish was determined.

3・1・4 Inspection of the sensitivity of fish: The inspection was carried out according to Tabata's report (Tabata, 1972). The death rate of fish in 0.5 ppm of mercuric chloride (as Hg) should not be more than 20% while that in 1.0 ppm should be more than 20% after 24 hours.

3・2 Determination of partition coefficient between *n*-octanol and water.

3・2・1 Test solution : Each substance was dissolved in distilled water or *n*-octanol at the concentration of 100 mg/liter.

3・2・2 Operating conditions: Estimation was carried out according to OECD Chemicals Testing Programme Ecotoxicology Group (OECD Chemicals Testing Programme Ecotoxicology Group, 1979), i. e., 100 ml of aqueous test solution was shaken with 100 ml of *n*-octanol in a separatory funnel at 25°C for 2 hours and then stood for 2 hours. One hundred of *n*-octanol test solution was shaken with 100 ml of water in the same manner. After separating into two phases, concentration of organic nitrogen compound was determined by gas liquid chromatography while that of dye was determined by colorimetry.

The partition coefficient (P) is defined as the ratio of the equilibrium concentration (C) of a dissolved substance in a two-phases system consisting of two almost immissible solvent (n-octanol and water).

$$P=\frac{\text{Cn-octanol}}{\text{Cwater}}$$

3・3 Determination of accumulated dye to the gills of fish

Carps were exposed to dye solution (1,000 mg/liter) for 30, 60 and 180 minutes and then the gills and intestines were taken out by dissection, then the accumulated dye was eluted with 70% ethanol containing 1% ammonia water, determined spectrophotometrically and expressed as amount of accumulated dye on each organ.

3・4 Determination of dissolved oxygen in dye solution

A 1 liter flask was filled with dye solution (50 mg/liter) to keep 10 Himedaka in it. Electrode sensor of dissolved oxygen meter was inserted into the neck of the flask and the flask was kept at 25°C and occasionally mixed. Dissolved oxygen content in the flask was checked periodically.

3・5 Determination of red corpuscles in blood

Carps were dipped in Rose Bengale solution (100 mg/liter) or in water added with dry ice to evolve oxygen. After the fish had been dead, 1 ml of blood was taken out and mixed with 0.1 ml of 1 M EDTA dipotassium salt solution (antisolidifying agent) and

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then the red corpuscles were counted by Coulter counter.

RESULTS AND DISCUSSION

1. Results of TLm values and partition coefficient

TLm values and partition coefficients of 16 kinds of aliphatic nitrogen compounds, 28 kinds of aromatic nitrogen compounds and 37 kinds of dyestuffs are as shown in Table 1, 2, and 3.

Table 1. TLm values and partition coefficients of aliphatic amines, amides and nitriles

	Substance	TLm (mg/liter)[a) (24 hr)	(48 hr)	Partition coefficient between *n*-octanol and water
1	Methylamine	1,000	1,000	0
2	Dimethylamine	1 ,000	1,000	0
3	Trimethylamine	1,000	1,000	0
4	Ethylamine	1,000	1,000	0
5	Diethylamine	1,000	1,000	0
6	Triethylamine	1,000	720	0
7	*n*-Propylamine	1,000	1,000	0
8	*iso*-Propylamine	1,000	1,000	0
9	*n*-Butylamine	1,000	1,000	0
10	Ethylenediamine	1,000	1,000	0.05
11	Diethylenetriamine	1,000	1,000	0.11
12	Ethylcarbamate	1,000	1,000	2,77
13	N, N-Dimethylformamide	1,000	1,000	0.13
14	N, N-Dimethylacetamide	1,000	1,000	0.16
15	Acetonitrile	1,000	1,000	0.29
16	Acrylonitrile	50	32	2.00

a) Ten fish of Himedaka were used in 2 liter of the solution.

With respect to the aliphatic nitrogen compounds investigated, shown in Table 1, 87.5% were low in toxicity, their TLm values being not less than 1,000 mg/liter, while their partition coefficients were less than 3. Contrary to this, 92.8% of the aromatic nitrogen compounds investigated had TLm values of less than 50 mg/liter (Table 2), indicating high in toxicity to fish. On regarding to partition coefficient, 14.3% of them (diphenylamine, 3, 5-xylidine etc.) showed the values of more than 1,000. It was concluded that the larger the solubility of a chemical substance in *n*-octanol was, the larger was the toxicity to fish.

With respect to dyestuffs, 24.3% of their TLm values were not less than 1,000 mg/liter, while 52.6% less than 50 mg/liter (Table 3). Besides, 78.4% of their partition coefficients were less than 50 and 13.5% of them were more than 100.

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Table 2. TLm values and partition coefficients of aromatic nitrogen compounds

	Substance	TLm (mg/liter)[a] (24 hr)	(48 hr)	Partition coefficient[b]
1	Aniline	74.0	48.0	7.50
2	N-Methylaniline	50.0	38.0	60.00
3	*p*-Toluidine	60.0	42.0	9.53
4	N, N-Dimethylaniline	56.0	33.0	415.67
5	N, N-Dimethyl-*p*-toluidine	44.0	20.0	924.93
6	3, 5-Xylidine	35.0	17.0	1,100.00
7	*p*-Ethylaniline	60.0	32.0	107.10
8	N-Ethylaniline	71.0	33.0	82.30
9	N, N-Diethylaniline	40.0	25.0	1,491.54
10	Phenylhydrazine	21.4	15.7	20.20
11	*p*-Phenylenediamine	25.0	20.0	2.33
12	*p*-Toluylenediamine	16.0	12.0	3.80
13	*p*-Anisidine	55.0	40.0	12.79
14	*p*-Chloroaniline	43.0	28.0	2.57
15	*p*-Nitroaniline	68.0	50.0	3.00
16	Benzonitrile	27.0	15.0	49.00
17	Phthalodinitrile	42.5	27.0	3.82
18	*iso*-Phthalonitrile	40.0	22.0	2.50
19	Phthalimide	30.0	16.5	3.53
20	Nitrobenzene	24.0	20.0	39.00
21	*p*-Nitrophenol	12.0	7.0	11.82
22	Picric acid	210.0	117.0	1.00
23	Benzidine	16.5	10.5	101.60
24	Diphenylamine	4.0	2.2	4,165.67
25	α-Naphtylamine	15.0	7.0	168.49
26	α-Nitroso-β-naphthol	0.6	0.4	1,100.00
27	Pyridine	400.0	330.0	5.03
28	2-Aminopyridine	12.0	6.0	3.00

a) Ten fish of Himedaka were used in 2 liter of the solution.
b) Partition coefficient between *n*-octanol and water.

2. Correlation between TLm value and partition coefficient

In 1977, Freed et al. (1977) found proportional relationship between partition coefficient and insecticidal activity on organophosphorus pesticides. In 1978, Sugiura et al. (1978) studied on the accumulation of mono-, di-, and tetrachlorobiphenyls as well as di-, tri-, and tetrabromobiphenyls in the Himedaka and found that their accumulation were proportional to their partition coefficient between n-octanol and water.

In this report, the correlation between TLm values (toxicity to fish) and partition coefficients of organic nitrogen compounds were studied. The results on aniline derivatives and other nitrogen compounds are indicated in Fig. 1 and 2.

With respect to aniline derivatives except p-chloroaniline, p-phenylenediamine and

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Table 3. TLm values and pertition coefficients of dyestuffs

	Substance	TLm (mg/liter)[a] (24 hr)	(48 hr)	Partition[b] coefficient
1	Azobenzene	1.0	0.5	36.00
2	*p*-Aminoazobenzene	1.7	0.7	31.00
3	*p*-Dimethylaminoazobenzene	1.1	0.6	106.00
4	2, 4-Diaminoazobenzene	0.5	0.3	52.00
5	Oil Yellow AB	0.9	0.5	31.26
6	Oil Yellow OB	0.6	0.4	50.50
7	Metanil Yellow	90.0	68.0	5.00
8	Bismark Brown	0.8	0.5	100.50
9	Auramine	4.4	3.2	8.10
10	Fuchsine	6.8	4.3	4.56
11	Malachite Green	0.6	0.3	5.60
12	Crystal Violet	0.2	0.1	24.64
13	Indigo	63.0	42.0	110.00
14	Methylene Blue	18.0	13.0	0.06
15	Safranine T	12.0	7.0	0.33
16	Induline	1,000.0	1,000.0	0.67
17	Martius Yellow	1.4	0.8	2.50
18	Naphthol Yellow S	1,000.0	1,000.0	0.02
19	Orange I	1,000.0	1,000.0	2.81
20	Orange II	1,000.0	1,000.0	3.74
21	Naphthol Black B	1,000.0	1,00.0	1.28
22	Congo Red	1,000.0	1,000.0	5.85
23	Quinoline Yellow	1,000.0	1,000.0	0.05
24	Naphthol Green B	1,000.0	1,000.0	0.08
25	Rhodamine B	17.0	12.0	74.00
26	Cyanine	0.7	0.4	5.25
27	Indophenol	0.9	0.5	150.00
28	Indanthrene	72.0	46.0	0.18
29	Alizarine	2.1	1.1	120.00
30	Alizarine Red S	250.0	170.0	0.02
31	Light Green SF	1,000.0	1,000.0	0.21
32	Guinea Green B	1,000.0	990.0	0.27
33	Acid Violet 6B	950.0	760.0	0.30
34	Eosine	1,000.0	620.0	0.11
35	Erythrosine	710.0	340.0	0.13
36	Phloxine	280.0	200.0	0.18
37	Rose Bengale	130.0	100.0	0.35

a) Ten fish of Himedaka were used in 2 liter of each solution.
b) Partition coefficient between *n*-octanol and water.

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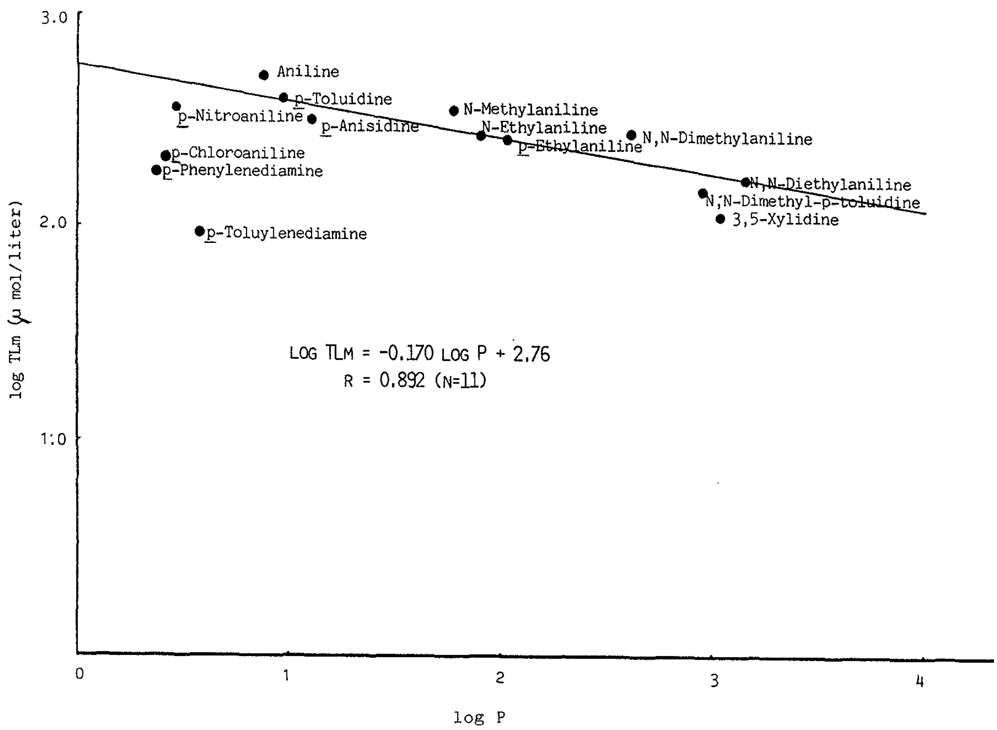

Fig. 1. **Correlation between Partition Coefficient and TLm of Aniline Derivatives**
P : Partition coefficient

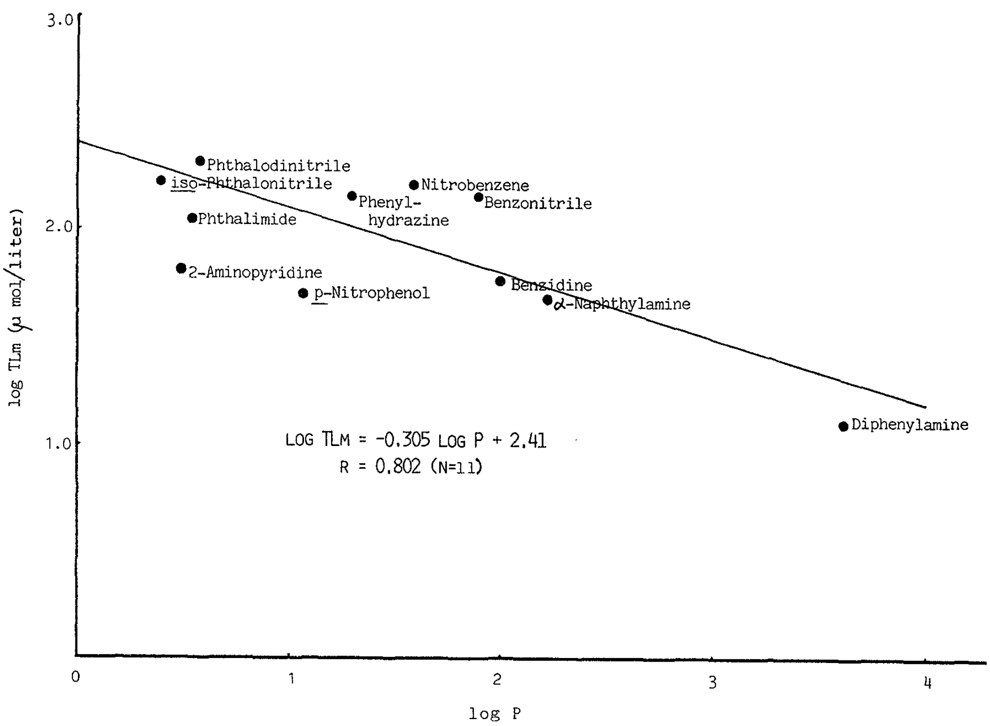

Fig. 2. **Correlation between Partition Coefficient and TLm of aromatic Compounds**
P : Partition coefficient

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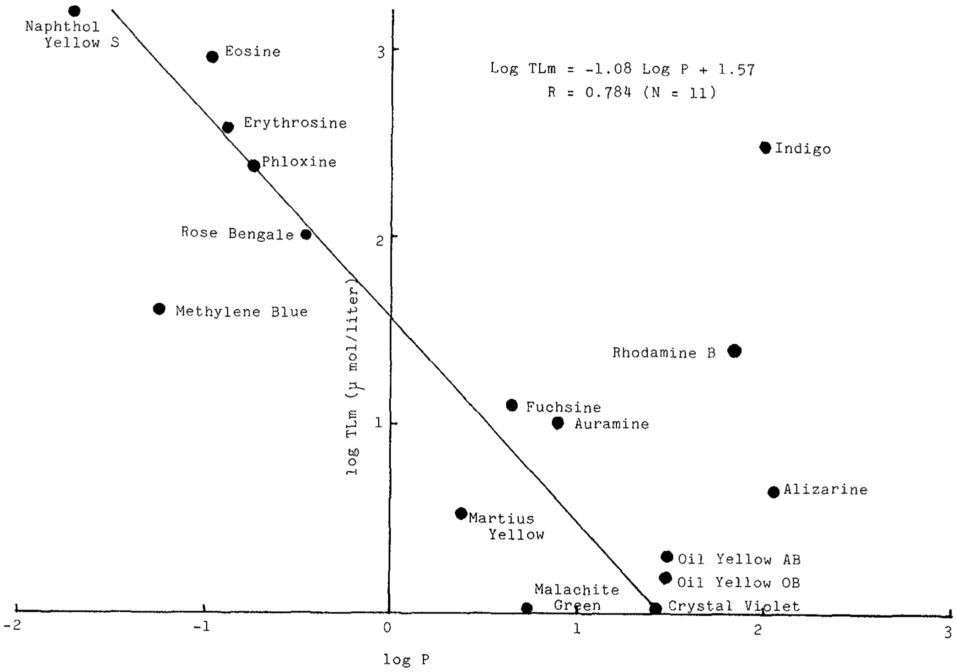
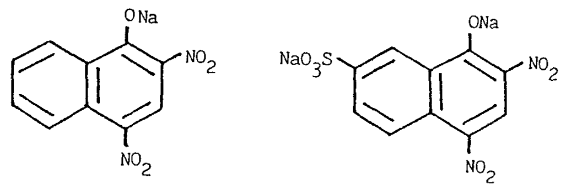


Fig. 3. Correlation between partition Coefficient and TLm of Dyestuffs
P : Partition coefficient



	Martius Yellow	Naphthol Yellow S
TLm value:	less than 1 mg/liter	not less than 1,000 mg/liter
Partition coefficient:	2.50	0.02

Fig. 4. Chemical Structures of Martius Yellow and Naphthol Yellow S

p-toluylenediamine, the larger the partition coefficient was, the smaller was TLm value (Fig. 1). On the whole, good relationships were observed between TLm values and partition coefficients of aniline derivatives and other nitrogen compounds excluding a few compounds. The relationship between TLm values and partition coefficients of dyestuffs was also correlative except a few dyes (Fig. 3). The TLm value and the partition coefficient of Martius Yellow (C. I. 10315) (MY) were compared with those of Naphthol Yellow S (C. I. 10316) (NYS) having similar chemical structure (Fig. 4). MY has a relatively large partition coefficient, 2.50, and its toxicity to fish is very high, TLm value being less than 1 mg/liter. Contrary to this, NYS has a very small partition coefficient of 0.02 and its toxicity to fish is very low, TLm value being not less than 1,000 mg/liter. The replacement of aromatic hydrogen by sulfonate group makes the

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dye more hydrophilic and lower the partition coefficient, which results in the less accumulation of the dye in the fish body. From these results, it is supposed that the hydrophobic properties of compounds indicated by partition coefficient are related to the toxicity.

Consequently, it is considered that the toxicity to fish of almost all of aromatic nitrogen compounds and dyes is owing to passage through their membrane.

3. Acute toxicity of Methylene Blue (MB) and Rose Bengale (RBL)

3・1 Accumulation to fish

Although MB and RBL have a small partition coefficient, their toxicity to fish is relatively high (Fig. 3). It is assumed that their toxicity to fish is owing to different reason from the passage through their membrane.

It was observed that their gills were dyed deeply, when fish died after being kept in the solution of MB and RBL. It was assumed that dyes might have been bound with the components of the gills tissues. In order to make clear the relation between toxicity and the affinity of these dyes to gills, amount of accumulated dyes to gills and to intestine were examined by use of carps (Table 4).

Table 4. Time course of accumulated dyes at gill and intestine of fish

Dye	Time (min)	Amount of accumulated dye ($\mu g/g$)		Number of fish died
		Gill	Intestine	
Rose Bengale	30	1.0	0	0/10
	60	5.4	0	2/10
	180	73.5	0	5/10
Methylene Blue	30	2.1	0	1/10
	60	8.6	0	3/10
	180	105.8	0	7/10

Note. 1) Fish (carp) was exposed to a solution of dye (1,000 mg/liter).
2) Dye accumulated to organ was eluted and determined by spectrophotometry.

The amount of MB and RBL accumulated to the gills increased with the lapse of time, while they were not found in the intestine until 180 minutes. From these results, it is assumed that the toxicity of MB and RBL was owing to disturbance of breathing of fish by accumulation of them to the gills.

3・2 Oxygen consumption by fish in MB and RBL solutions

In order to make clear the disturbance of fish breathing by MB and RBL, oxygen consumption by fish in the MB and RBL solutions were compared with that in water as the control. The results are shown in Fig. 5. Decrease ratios of dissolved oxygen in MB and RBL solutions were smaller than that in control, indicating that the fish uptook extremely few amount of dissolved oxygen in dye solution. It is assumed that high affinity of MB and RBL to gills is supposed to be responsible to depressing the function of gills and that the death of fish might be due to anoxemia.

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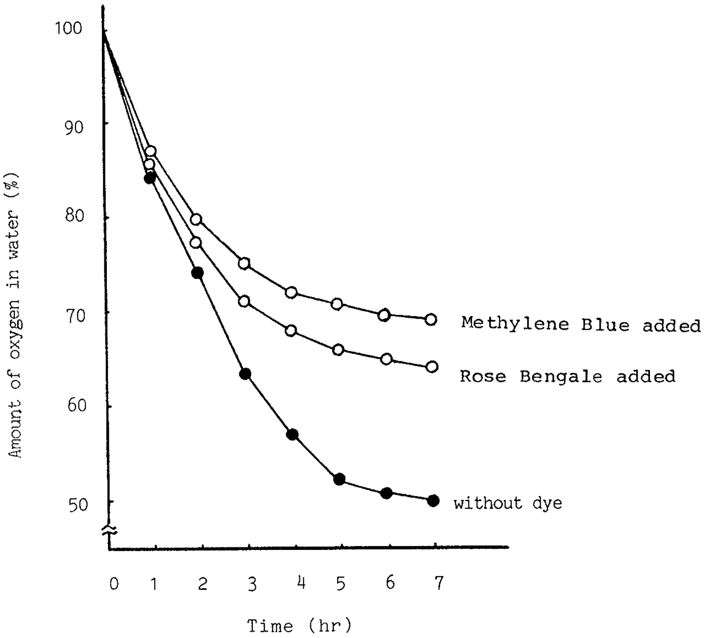

Fig. 5. Time Course of Oxygen Consumption by Fish in Dye Solution
Note. 1) Ten fish of Himedaka were kept in 1 liter of 50 mg/liter dye solution.
2) Dissolved oxygen in dye solutions were determined by DO meter.

Table 5. Number of red corpuscles of fish kept under three different conditions

	Exposed solution	Number of red corpuscle ($\times 10^4$/ml)	
A.	Water	80	(Fish alive)
B.	Rose Bengale (1,000 mg/liter)	130	(Fish died)
C.	Water added with dry ice to evolve dissolved oxygen	120	(Fish died)

Note. Fish (carp) was exposed to each solution, and the number of red corpuscle in fish blood was determined by Coulter counter after 24 hours in case of A and immediately after fish had died in case of B and C.

3・3 Number of red corpuscles in fish blood

It was already recognized that number of red corpuscles in fish blood increased rapidly with the decrease in oxygen in the medium (Kawamoto, 1970). The number of red corpuscles of carp kept in the dye solution was investigated (Table 5). The number of red corpuscles in water was 80×10^4/ml, while the number in RBL solution increased to 130×10^4/ml and of the same order with the number of 120×10^4/ml in blood of fish kept in water without oxygen by the addition of dry ice. Consequently, it was assumed that fish became anoxemic and died by the accumulation of RBL to the gills, resulting in the depression of gills function.

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CONCLUSION

TLm test by use of Himedaka was carried out on 16 kinds of aliphatic nitrogen compounds, 28 kinds of aromatic compounds and 37 kinds of dyestuffs containing nitrogen, and the TLm values were compared with the partition coefficients of these compounds between *n*-octanol and water. The following results were obtained.

1. The toxicity of aliphatic nitrogen compounds to fish were low and their TLm values were not less than 1,000 mg/liter. Contrary to this, aromatic nitrogen compounds were high in toxicity and their TLm values were less than 50 mg/liter.

2. Aromatic nitrogen compounds had a good correlation between TLm values and partition coefficients indicating that the larger the solubility of compounds in *n*-octanol was, the larger was the toxicity to fish.

3. Dyestuffs had a correlation between TLm values and partition coefficients. From the results of the comparison of MY and NYS, introduction of hydrophilic substituent such as sulfonate group to the chemical structure makes the solubility of compound in *n*-octanol lower as well as its toxicity.

4. MB and RBL had high acute toxicity to fish. It was found that these dyes are accumulated to gills of fish to prevent the uptake of dissolved oxygen in water by fish and make fish suffer from anoxemia and die.

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